Local Suppression of Contact Hypersensitivity in Mice by a New Bifunctional Psoralen, 4,4′,5′-trimethylazapsoralen, and UVA Radiation

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Although psoralens plus UVA radiation (320–400 nm) have been widely used for the treatment of dermatologic diseases, the toxic effects of these agents have led investigators to develop new photochemotherapeutic compounds. One such compound is 4,4′,5′-trimethylazapsoralen (TMAP), a new bifunctional molecule. The purpose of this study was to examine the immunologic side effects of repeated treatment of C3H mice with TMAP plus UVA radiation. During this treatment, the number of ATPase$^+$, Ia$^+$, and Thy-1$^+$ dendritic epidermal cells greatly decreased in the treated site, despite the lack of phototoxicity. The reduction in the number of detectable cutaneous immune cells was accompanied by a decrease in the induction of contact hypersensitivity to dinitrofluorobenzene applied to the treated skin, an impairment in the antigen-presenting activity of draining lymph node cells, and the presence of suppressor lymphoid cells in the spleen of unresponsive mice. Treatment with UVA radiation alone also reduced the number of ATPase$^+$, Ia$^+$, and Thy-1$^+$ cells in the skin, but did not cause any detectable alterations in immune function. This implies that morphologic alterations in these cells do not necessarily indicate loss of function. Thus, although TMAP in combination with UVA radiation is not overtly phototoxic, it is highly immunosuppressive in mice. *J Invest Dermatol 97:50–54, 1991*

The linear, bifunctional psoralen, 8-methoxypsoralen (8-MOP), has been extensively used with UVA (320–400 nm) radiation for the treatment of several dermatologic diseases such as psoriasis and cutaneous T-cell lymphomas [1]. The therapeutic effectiveness of treatment with 8-MOP plus UVA radiation (PUVA) is thought to correlate with the formation of lesions in DNA of the epidermal cells [2]. On the molecular level, two classes of psoralens can be distinguished: linear, bifunctional psoralens (e.g., 8-MOP), which form both monofunctional and bifunctional adducts (interstrand crosslinks) with pyrimidine bases in DNA and angular, monofunctional psoralens (e.g., angelicin), which can form only monofunctional adducts with pyrimidine bases of DNA following UVA exposure. Although PUVA treatment has been shown to be therapeutically active in the management of cutaneous hyperproliferative diseases, the toxic effects of these compounds have led physicians to seek new photochemotherapeutic agents. One such agent is 4,4′,5′-trimethylazapsoralen (TMAP), a new bifunctional compound that exhibits high antiproliferative activity but lacks phototoxic effects [3,4].

In a previous study we demonstrated that TMAP plus a low dose of UVA radiation induced morphologic alterations of cutaneous immune cells and decreased the number of detectable Langerhans (ATPase$^+$, Ia$^+$ cells) and Thy-1$^+$ cells in the epidermis of BALB/c mice. These effects occurred after three treatments per week given for 4 weeks, even though no evidence of phototoxicity was noted either grossly or histologically [4]. The purpose of this study was to determine whether the same regimen of TMAP plus UVA radiation also produced local immunosuppressive effects like those observed after 8-MOP and UVA radiation (PUVA) or whether the reduced phototoxicity would be accompanied by a decrease in immunosuppressive activity.

To test the immunologic effects of TMAP plus UVA radiation, C3H mice were contact sensitized with dinitrofluorobenzene (DNFB) through the treated skin. Repeated treatment of mice with TMAP plus UVA decreased the contact hypersensitivity (CHS) response to DNFB, impaired the antigen-presenting activity of cells in the lymph nodes (DLN). draining the site of sensitization, and induced suppressor lymphoid cells in the spleen.

MATERIALS AND METHODS

Animals Specific pathogen-free female C3H/HeN (MTV$^-$) mice were obtained from the NCI-Frederick Cancer Research Facility Animal Production Area (Frederick, MD). Age-matched, 9- to 12-week-old mice were used for these experiments. The mice received NIH-31 open formula mouse chow and sterile water ad libitum. Ambient lighting was controlled to provide regular 12-h light, 12-h dark cycles.

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Abbrevations:
CHS: contact hypersensitivity
DLN: draining lymph nodes
DNFB: 2,4-dinitrofluorobenzene
DTH: delayed type hypersensitivity
IFP: intrafootpad
8-MOP: 8-methoxypsoralen
PUVA: 8-MOP plus UVA radiation
TMAP: 4,4′,5′-trimethylazapsoralen
UVA: ultraviolet A radiation (320–400 nm)

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Radiation Sources UVA radiation (320–400 nm) was provided by a Dermalight 2001 with an optical H1 filter (Dermalight Systems, Studio City, CA).The spectral irradiance was measured by an Optronics 742 spectroradiometer (Optronic laboratories Inc, Orlando, FL). The peak emission of the lamp occurred at 366 nm, and 99.5% of the energy emitted was between 320 and 400 nm. The average incident dose rate was 38.5 W/m^2.

Treatment Protocol The bifunctional psoralen 4,4′,5′-trimethylazapsoralen (TMAP), obtained as a crystalline powder, was dissolved in 70% alcohol to a concentration of 430 μg/ml. The solution was kept shielded from ambient light. The dorsal hair of the mice was removed once a week with electric clippers. The drug was applied topically in a 300-μl volume (129 μg) on the shaved dorsal skin of the mice, and 45 to 60 min thereafter, the mice were exposed to 10 kJ/m^2 of UVA radiation. Ten minutes before irradiation, all mice, including non-irradiated groups, were anesthetized by intraperitoneal (IP) injection of sodium pentobarbital (50 mg/kg body weight). Mice were placed 22 cm below the radiation source in a plastic cage separated into individual chambers. The ears were shielded with aluminum foil during the UVA irradiation. This treatment was given three times a week for 4 weeks. To compare the effects of TMAP and 8-MOP, we used equimolar doses of 8-MOP (Sigma Chemical Co, St. Louis, MO) applied in a 300-μl volume of 70% ethanol, followed by the same dose of UVA radiation (PUVA). Mice that received the drugs or alcohol alone, UVA radiation, or alcohol plus UVA radiation served as controls.

Enumeration of Cutaneous Immune Cells At the end of the treatment period, mice were killed and their dorsal skin was removed. The connective tissue underlying the skin was removed with a scalpel blade, and the epidermis was separated from the dermis after a 2-h incubation at 37°C in buffered EDTA [5]. The epidermal sheets were fixed and then stained in order to detect ATPase$^+$, Ia$^+$, and Thy-1$^+$ cells. The numbers of dendritic epidermal cells were determined using a Nikon Optiphot microscope (Nikon Inc., Garden City, NY). Between 10 and 20 fields were counted from each experimental group. The cells were counted at ×400 magnification using a WHK 10 × 20L optical grid (Olympus Optical Co, Tokyo, Japan), and the mean number of cells/mm^2 in each group was calculated. The mean number of dendritic epidermal cells was then expressed as a percentage of the control value.

Induction of CHS The dorsal (treated) skin of the mice was painted with 50 μl of 0.3% dinitrofluorobenzene (DNFB) in acetone (v/v), 24 h after the last treatment. The mice were tested for CHS 6 d later by applying 5 μl of 0.2% DNFB on each surface of both ears, which had been shielded from UVA radiation. Ear thickness was measured with a spring-loaded micrometer before and 24 h after application of the challenge dose.

Transfer of Suppression of CHS After the CHS response was measured, mice were killed, and single-cell suspensions were prepared from their spleens. These cells were injected intravenously (IV) into normal syngeneic mice at a concentration of 10^8 viable, nucleated cells in 1 ml Eagle's minimal essential medium (Gibco, Grand Island, NY). These mice were immediately sensitized on shaved abdominal skin with 50 μl of 0.3% DNFB in acetone (v/v) and challenged 6 d later on the ears as described above.

Antigen-Presenting Activity of DLN Cells Twenty-four hours after the last treatment, the dorsal skin of the mice was painted with 50 μl of 0.3% DNFB in acetone, and 18 h later, a single-cell suspension was prepared from the inquinal, axillary, and subscapular lymph nodes. The cells were washed once in Eagle's minimal essential medium and filtered through nylon mesh. Then, 0.05 ml containing 10^6 cells was injected into each hind footpad (ifp) of syngeneic recipients. The recipients were challenged on the ears with 0.2% DNFB 6 d later, as described above. The ability of the DLN cells to induce CHS in recipient mice is due to the presence of Ia$^+$, antigen-bearing dendritic cells [6–8].

Statistical Analysis Student two-tailed t test was used to test the significance of differences between control and treated groups.

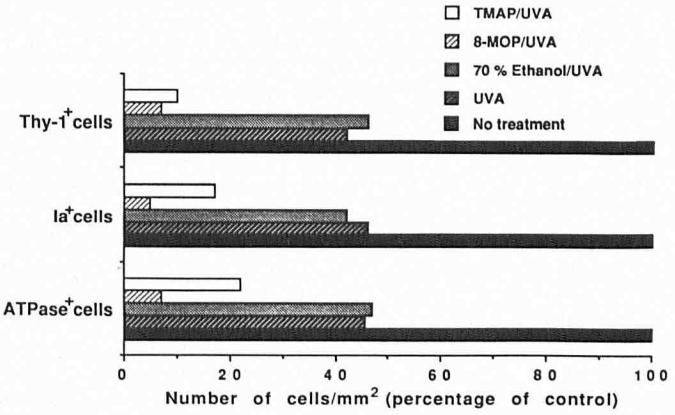

Figure 1. Effect of treatment on the number of cutaneous immune cells. C3H mice were treated 3 times a week for 4 weeks. The number of cells was determined 24 h after the last treatment. Mice in the control group were untreated. Equimolar doses of 8-MOP and TMAP were topically applied on shaved back skin of C3H mice, which were then irradiated with 10 kJ/m^2 of UVA radiation. All the values are significantly different from the untreated controls ($p < 0.001$).

RESULTS

Phototoxicity Mice treated with 8-MOP and UVA radiation exhibited severe, gross cutaneous phototoxicity, including erythema and eventual ulceration, whereas equivalent treatment with TMAP and UVA radiation caused no visible changes in the skin. Histologic examination of the treated skin did not reveal any inflammatory cells in the dermis. The absence of parakeratosis in the stratum corneum and epidermal acanthosis added further evidence that TMAP had little phototoxicity [4]. In addition, no toxicity was observed in skin treated with either UVA or drugs alone, vehicle plus UVA radiation, or vehicle alone.

Effect of Treatment on the Number of Cutaneous Immune Cells As shown in Fig 1, at the end of the treatment period mice that had received UVA radiation or alcohol plus UVA radiation had a decreased number of dendritic epidermal cells compared to the non-treated mice. The addition of either 8-MOP or TMAP to the UVA radiation caused further reductions in the number of ATPase$^+$, Ia$^+$, and Thy-1$^+$ cells, as we reported previously [4]. However, mice treated with PUVA had significantly fewer ATPase$^+$ and Ia$^+$ cells than those treated with TMAP plus UVA ($p < 0.05$).

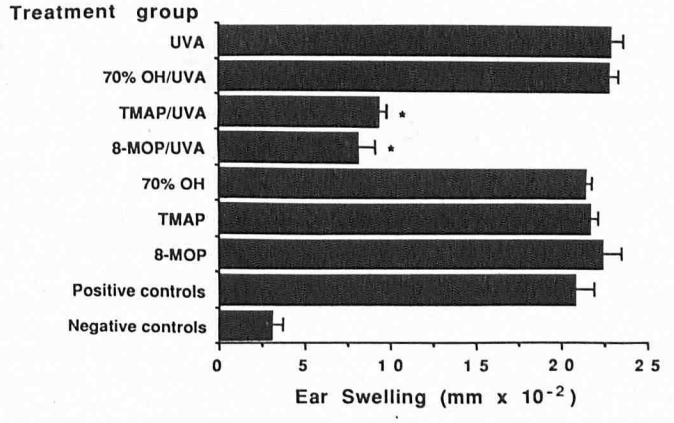

Figure 2. CHS response to DNFB in treated mice. Twenty-four hours after the last treatment, the mice were sensitized with 50 μl of 0.3% DNFB in acetone on the treated back skin and challenged 6 d later on both ear surfaces with 5 μl of 0.2% DNFB in acetone. Positive controls were sensitized but not treated; negative controls were untreated and unsensitized. *, $p < 0.001$ versus the positive controls.

Therefore, treatment with TMAP and UVA radiation appeared to have a lesser effect on the number of Langerhans cells than PUVA treatment. No significant difference was found in the number of Thy-1$^+$ cells between these two treatment groups.

Effect of Treatment on the CHS Response Mice given the various treatments were sensitized with DNFB through the treated skin and challenged 6 d later on the ears, which had been protected during UVA irradiation. Mice treated with the drugs or UVA alone or alcohol and UVA exhibited a normal CHS response to DNFB applied on the treated site (Fig 2). Thus, the 50% decrease in the number of ATPase$^+$ and Ia$^+$ cells in the mice treated with UVA alone or alcohol and UVA did not affect the ability of the animals to be sensitized. In contrast, mice treated with PUVA or TMAP plus UVA radiation demonstrated a profound suppression (72% and 65%, respectively) of CHS; no significant difference was found between these groups in the CHS response.

Transfer of Unresponsiveness with Spleen Cells To examine whether the decreased CHS response to DNFB was associated with the presence of suppressor cells, mice were sensitized through the treated skin and challenged 6 d later. Spleen cells (1×10^8) from hyporesponsive mice were injected iv into normal mice, which were then immediately sensitized by epicutaneous application of DNFB. Transfer of spleen cells from 8-MOP or TMAP plus UVA-treated, DNFB-sensitized donors suppressed the induction of CHS to DNFB in recipient mice (Fig 3), indicating that suppressor lymphoid cells were present in the spleen cell suspensions. No significant difference was seen in the suppressive activity transferred by spleen cells from these two treatment groups.

Activity of DLN Cells We then tested whether the decreased CHS response to DNFB was associated with an alteration in the antigen-presenting activity of cells in the DLN. As shown in Fig 4, DLN cells from untreated mice sensitized with DNFB induced CHS when injected into the footpads of normal recipients. This activity was markedly reduced in mice treated with 8-MOP or TMAP plus UVA radiation before sensitization. No significant difference was observed in the alteration of the antigen-presenting activity between these two groups. DLN cells from mice treated with the drugs or UVA radiation alone and sensitized with DNFB showed normal antigen-presenting activity. This experiment demonstrates that antigen-presenting cells are functionally altered in mice treated with 8-MOP or TMAP plus UVA radiation.

Comparison of UVA Versus TMAP Plus UVA The ability of UVA radiation alone to reduce the numbers of ATPase$^+$, Ia$^+$, and Thy-1$^+$ cells in the epidermis without decreasing CHS and inducing suppressor lymphoid cells in the spleen could have two explanations. First, a 50% reduction in the numbers of these cells might be insufficient to cause decreased function. Alternatively, the morphologic alterations may not always correlate with altered function, and therefore other qualitative alterations in the cells may be required for altered function. To distinguish between these possibilities, we compared the effects of UVA alone and TMAP plus UVA on the activity of DLN cells under conditions where they caused similar reductions (approximately 50%) in the number of cutaneous immune cells. As shown in Fig 5, a dose of TMAP plus UVA radiation sufficient to cause a 50% reduction in the number of ATPase$^+$, Ia$^+$, and Thy-1$^+$ cells also caused a significant reduction in the antigen-presenting activity of the DLN cells. In contrast, UVA treatment did not alter the activity of the DLN cells, even though similar reductions in the numbers of ATPase$^+$, Ia$^+$, and Thy-1$^+$ cells were

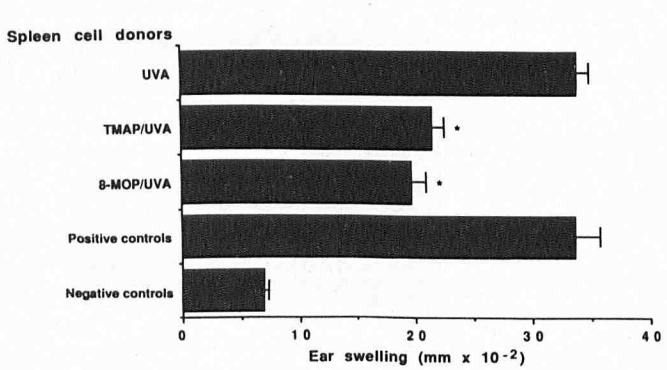

Figure 3. Transfer of suppression of CHS with spleen cells. 10^8 viable spleen cells from hyporesponsive mice were injected iv into normal syngeneic mice. Recipients of spleen cells were immediately sensitized on shaved abdominal skin with 50 μl of 0.3% DNFB in acetone and challenged 6 d later on both ear surfaces with 5 μl of 0.2% DNFB in acetone. Negative controls were unsensitized, and positive controls were injected iv with spleen cells from untreated mice prior to sensitization with DNFB. *, $p <$ 0.001 versus the positive controls.

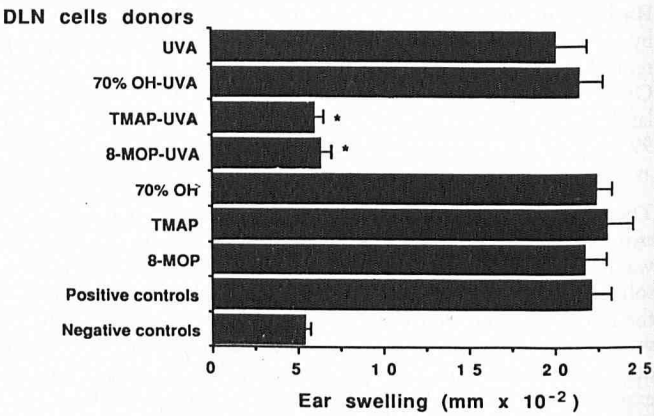

Figure 4. activity of antigen-presenting DLN cells. Twenty-four hours after the last treatment, the mice were sensitized through the treated skin with 50 μl of 0.3% DNFB, and 18 h later 2×10^6 DLN cells from mice given different treatments were injected ifp into normal syngeneic mice. The recipients were challenged 6 d later on both ear surfaces with 5 μl of 0.2% DNFB in acetone. The next day, the resulting ear swelling was measured. Negative controls were untreated and unsensitized. Positive controls were sensitized ifp with 2×10^6 DLN cells from untreated, DNFB-sensitized mice. *, $p < 0.001$ versus the positive controls.

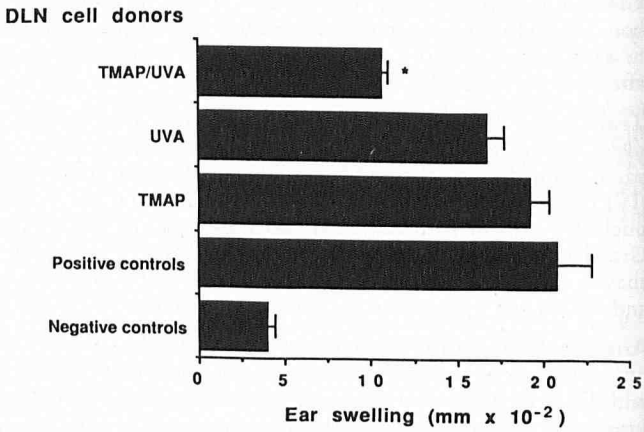

Figure 5. Activity of antigen-presenting activity DLN cells. Mice were treated three times a week for 2 weeks, with either TMAP alone (129 μg), or UVA alone (20 kJ/m^2), or TMAP (129 μg) plus UVA (10 kJ/m^2), in order to decrease by nearly 50% the number of epidermal dendritic cells. Twenty-four hours after the last treatment, the treated skin was sensitized with 50 μl of 0.3% DNFB, and 18 h later, 2×10^6 DLN cells were injected ifp into normal recipients, which were challenged 6 d later with 0.2% DNFB on the ears as described above. Negative controls were untreated and unsensitized; positive controls were sensitized ifp with DLN cells from untreated, DNFB-sensitized mice. *, $p < 0.001$ versus the positive controls.

observed. This result demonstrates that there is an important qualitative difference between the effects of UVA and TMAP plus UVA on the antigen-presenting cells, in addition to their effects on the number of these cells in the epidermis.

DISCUSSION

During the past 15 years, treatment with PUVA has been widely used for the treatment of cutaneous diseases such as psoriasis, vitiligo, and mycosis fungoides. However, this treatment is associated with toxicity in patients and is also immunosuppressive. Studies in humans and animals have demonstrated that dendritic epidermal cells are reduced in number and morphologically altered following PUVA treatment [9,10]. In addition, PUVA treatment reduces CHS to contact sensitizers applied through irradiated skin [11] or unirradiated skin in mice [12] and in humans [13]. Because the suppression was reversed by cyclophosphamide treatment, Horio and Okamoto [14] suggested that suppressor cells may be involved. Results from our laboratory have confirmed that splenic suppressor cells are induced after topical psoralen and UVA treatment [15]. Alteration of T-cell subsets and lymphocyte function have also been observed in patients treated with PUVA [16]. Because there is a greatly increased risk of cutaneous cancers in immunosuppressed patients [17,18], it is possible that the immunologic changes induced by PUVA treatment could play a role in the induction of skin cancers in PUVA-treated patients. The carcinogenic effect of PUVA in mice is well documented [19,20], but whether it induces skin cancer in humans is still being debated. Although Henseler et al [21] and Torinuki et al [22] observed no increase in the incidence of skin cancers, Stern et al [23] recently showed that long-term exposure to PUVA substantially increases the risk of squamous cell carcinoma. These problems have led investigators to develop new psoralen compounds in the hope of retaining the therapeutic activity while reducing the side effects.

Recently, azapsoralens, a new class of psoralen derivatives, have been synthesized [24]. One of these compounds, 4,4′,5′-trimethylazapsoralen (TMAP) is a linear, bifunctional furocoumarin that strongly inhibits DNA synthesis in Ehrlich's ascites cells [3], but lacks the phototoxic activity of 8-MOP, at least in the skin of laboratory rodents [3,4]. Like 8-MOP, TMAP binds to DNA, but has a stronger absorption capacity in the UVA region [3]. TMAP forms bifunctional adducts with DNA following UVA irradiation, but at a much slower rate than 8-MOP [3]. The abilities of TMAP to generate singlet oxygen to mediate oxidation reactions, or to interact with cellular molecules other than DNA have not yet been determined. Thus, it is not clear why equimolar doses of TMAP and 8-MOP produce such disparate acute effects on skin. Even after repeated treatments with topically applied TMAP on the shaved back skin of mice followed by exposure to 10 kJ/m^2 of UVA radiation, no phototoxicity could be detected, either grossly or histologically. In contrast, mice treated with the same regimen of equimolar doses of 8-MOP plus 10 kJ/m^2 of UVA exhibited a severe, gross, phototoxic reaction, with ulceration and necrosis of the skin [4]. Similar results were obtained with both BALB/c [4] and C3H mice.

The present study, using C3H mice, demonstrated that TMAP plus UVA radiation decreased the number of ATPase$^+$, Ia$^+$ Langerhans cells, and Thy-1$^+$ dendritic cells in murine skin and caused morphologic alterations in the remaining cells. These alterations were accompanied by functional alterations in immunologic reactivity. After 4 weeks of treatment, a significant decrease in the number of cutaneous immune cells was observed in mice treated with TMAP plus UVA radiation. The ATPase$^+$ and Ia$^+$ cells appeared to be slightly less sensitive to TMAP plus UVA than to PUVA ($p < 0.05$); however, the small difference in sensitivity of Langerhans cells was not reflected in functional studies, as both treatments decreased to the same extent the immunologic responsiveness to DNFB applied at the treated site. Suppression of the CHS response was associated with the presence of suppressor lymphoid cells in the spleen of unresponsive mice, which transferred suppression to normal recipients. In addition to the decrease in the induction of CHS to DNFB, treatment with TMAP plus UVA radiation profoundly affected the antigen-presenting activity of DLN cells. After injection into normal mice, these cells were unable to induce CHS.

Our data are similar to those obtained by Alcalay et al [15], who found that treatment of mice with 8-MOP, a bifunctional, phototoxic agent, or angelicin, a monofunctional, non-phototoxic psoralen, plus UVA radiation significantly decreased the number of epidermal Langerhans cells and the induction of CHS to DNFB. Cell transfer studies indicated that suppressor cells were present in the spleens of the unresponsive mice. However, the antigen-presenting activity of DLN cells was not investigated. That study demonstrated that local suppression of CHS and suppressor cell induction occurred regardless of whether the psoralen formed bifunctional adducts with DNA and regardless of its ability to cause phototoxicity.

In our experiment we noticed that treatment of mice with UVA radiation alone decreased the number of cutaneous immune cells by 50%. However, this decrease had no functional significance for the CHS response, was not associated with the induction of suppressor cells in the spleen, and did not alter antigen-presenting activity of DLN cells. In a previous experiment, Alcalay et al [15] found a marginal, though statistically significant, suppressive activity of spleen cells from mice treated with UVA alone. The reason for the discrepancy with our results may be related to the use of different treatment protocols. In contrast, mice treated with TMAP and UVA radiation over a 2-week period (six treatments) in order to obtain the same reduction in the number of epidermal dendritic cells demonstrated defective antigen-presenting activity of their DLN cells. Thus, despite a similar depletion of the cutaneous immune cells (50%), only mice treated with TMAP and UVA radiation exhibited altered antigen-presenting activity of their DLN cells. Our results therefore demonstrate that the morphologic alterations in dendritic epidermal cells observed after these treatments do not necessarily indicate altered function. Clearly, functional alterations, rather than simply a reduction in the number of detectable cutaneous immune cells, are required to bring about suppression of the CHS response. Conversely, several studies have demonstrated that the functional capacity of Ia$^+$ epidermal cells to present antigen could be dramatically altered despite normal expression of class II molecules [25,26].

In conclusion, our results show that despite the lack of phototoxicity, repeated treatments with TMAP plus UVA radiation have immunologic side effects on the CHS response similar to those produced by PUVA treatment. This conclusion should be considered in light of the proposed use of this new psoralen as a therapeutic agent in the treatment of dermatologic diseases. The mechanisms by which it exert its immunologic effects are as yet unclear. For many years, the biologic effects of psoralens were thought to result from their capacity to form photoadducts with DNA. However, recent studies provide increasing evidence that psoralens can react not only with nucleic acids, but also with membrane lipids and proteins [27–30]. The fact that certain monofunctional furocoumarins are as active as bifunctional psoralens in causing immune alterations implies that the formation of DNA crosslinks is not necessary to produce the immunologic effects. Furthermore, the finding that a bifunctional psoralen produces little or no detectable phototoxicity suggests that crosslinks are not responsible for the phototoxic effects of psoralens either. Recent studies demonstrate that two distinct types of photoreactions occur independently and concurrently when psoralen-treated skin is exposed to UVA radiation. One occurs in the absence of oxygen and involves the formation of covalent bonds mainly with pyrimidine bases in nucleic acids, but also with lipids, proteins, and receptor sites on membranes [27,28,31]. A second type of photoreaction is oxygen-mediated and involves the generation of singlet oxygen and radicals, which are able to damage the same targets [29,30,32]. Some studies have suggested that phototoxicity results from oxygen-dependent effects of psoralen plus UVA treatment [33]. Additional studies are required to determine whether the immunologic effects of psoralens also involve an oxygen-dependent pathway, or whether DNA damage is the triggering event.

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